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ABSTRACT

Physical Optics Corporation (POC) has investigated a new diagnostic technology that can be directly applied to the detection of the AIDS antigen or antibody, unlike the current procedures which only detect antibodies to HIV. POC's method is based on a highly sensitive ligand receptor, binding to its specific membrane receptor that is accompanied by conformational changes of the receptor protein which is followed by incorporating a fluorescent lipid probe into the target membrane. This provides a new opportunity for circumventing the well-known limitations of existing AIDS diagnostic tests which detect antibodies that confer non-protection, so all persons with antibodies are considered to have an active infection. In addition, a small percentage of infected persons have undetectable amounts of the antibody despite an established infection which is demonstrable by culturing the virus from tissues. Our proposed method of AIDS detection by polarization fluorometer for fluorescence anisotropy of lipid probe (PFFALP) offers direct proof of the presence of the AIDS virus. During Phase I, the feasibility of this method was achieved by using peripheral blood mononuclear cells (PBMC), and a combination of T-cell antigen-antibody. POC's improved PFFALP diagnostic test and a device based on this concept involving either viral antigen (AIDS) or antibodies will solve many of the problems of false positive and false negative tests associated with ELISA and Western Blot methods. This will be a first of its kind device for AIDS antigen detection that has a rapid response time, and is compact (portable) and low cost.

1.0 INTRODUCTION

Fabrication of a highly sensitive polarization fluorometer for fluorescence anisotropy of lipid probe (PFFALP) has been investigated by Physical Optics Corporation (POC) as a unique solution to the Army's need for screening military personnel for the AIDS virus. This proposed PFFALP device combines the specificity, sensitivity and compactness required in Army clinics for routine tests. In addition, the PFFALP device is capable of detecting both the AIDS virus and the AIDS antibody with the speed and accuracy lacking in currently available diagnostic methods. A breadboard model of the device, developed by POC under Phase I of this SBIR project, is shown in Figure 1-1.

Figure 1-1 Breadboard model of POC's PFFALP device.

The test is based on the use of fluorescent anisotropic lipid probe to evaluate the state of membrane under genome-antibody binding. The monitored phenomenon in this case is fluorescence depolarization of the light emitted by the probe [1].

Currently available test procedures designed to diagnose infection with the Human Immunodeficiency Virus (HIV) are highly sensitive and specific. Despite this, there is still a need for improvement. The best procedures detect antibodies to HIV. Unlike other infectious diseases,

these antibodies apparently confer no protection so all persons with antibodies are considered to have an active infection. The immune system requires time to make sufficient amount of antibody for detection so a variable "window" exists in which antibodies may be undetected in an infected person for six weeks to many months after infection. Some persons make incomplete and, consequently, non-diagnostic antibody responses during this time. In addition, it is well known that a few persons do not make detectable amounts of antibody despite long-established infection demonstrable by culturing virus from tissues.

The method of detection of viral antigen by PFFALP investigated in this program is a more sensitive indicator of infection than antibody detection. Such viral antigens should be present in persons who do not produce antibodies. Currently assays for the viral core p24 antigen are sometimes used for diagnostic purposes. This antigen, however, is not usually found in the serum until late in the course of HIV infection. It is not possible with the currently available methods to detect p24 antigen bound to circulating immune complexes.

Improved diagnostic tests involving either antibodies or viral antigen might solve many of the problems mentioned above. More sensitive PFFALP techniques of antibody detection should help to close the window and might detect the antibody in some of those who now remain seronegative. Similarly, more sensitive antigen detection techniques might make it possible to confirm HIV infection in patients with negative or doubtful serologic tests. This would be particularly likely if the antigen detection techniques could be combined with a method to detect viral antigen bound in immune complexes.

Moreover, new diagnostic techniques are urgently needed as surrogate markers of progress of infection. Trials of putative therapeutic agent currently must depend on serial CD4 counts, titer of infectious virus in the plasma, serum p24 antigen levels and non-specific indicators like serum levels of B2microglobulin and neoterin. A sensitive detection method for HIV antigen might make it much easier to assess the effects of treatment of the HIV infection.

The fluorescent probes used by Physical Optics Corporation (POC) are unique reagents with which POC has special expertise. The procedures to use them seems straightforward. Fluorescence polarization of the probe alters an immunological reaction involving a component of the cell membrane resulting in discrimination in the degree of polarization when viral antigen is present and when it is absent.

The ground- and excited-state electronic distributions of any fluorophore define directions within the molecule in which the probability of absorption or emission of a photon are greatest.[1] These

are the directions of the excitation and emission dipole moments, the magnitudes of which determine the maximum probabilities of absorption or emission. Figure 1-2 gives a geometrical perspective of fluorescence anisotropy. The fluorophore has excitation and emission dipoles that are parallel to the axis of symmetry. Vertically-polarized light (I_E) from the y-axis vertical polarizer selectively excites a population of fluorophores whose excitation dipoles are assumed to be symmetrically distributed about the z-axis. After a time, τ, photons (I_F) are emitted (electric vector parallel to the emission dipole) with probability $e^{-t/\tau}$ (τ = the excited state lifetime) and detected after analysis by the vertical and horizontal x-axis polarizers (not shown). Rotation of the probe during the time τ diminishes the vertical component (I_V) and enhances the horizontal component (I_H) of analyzed light in a way described by the time-dependence of the fluorescence anisotropy r(t). The assumptions of cylindrical probe symmetry and of symmetrical z-axis distribution simplify the description of r(t) to one involving an expansion of the angular distribution of probe concentration in terms of Legendre polynomials (P_n) in the cosine of the single angle θ. Thus, for relative concentration of fluorescent molecules emitting in a particular direction, we can write [2]

$$\frac{c}{c_o} = \frac{1}{3}\left[\mu^2 + \frac{1}{2}\left(3\mu_z^2 - \mu^2\right)\left(3\cos^2\theta - 1\right)\right]\Big/\mu_z^2 \qquad (1\text{-}1)$$

where μ_z is an absorption transition dipole matrix element and μ_z is its component. The condition of parallel excitation and emission dipoles allows for a single (P_2 approximation to describe the probe angular distribution) or triple ($P_2 + P_u$ approximation) do the angular distribution of r(t).

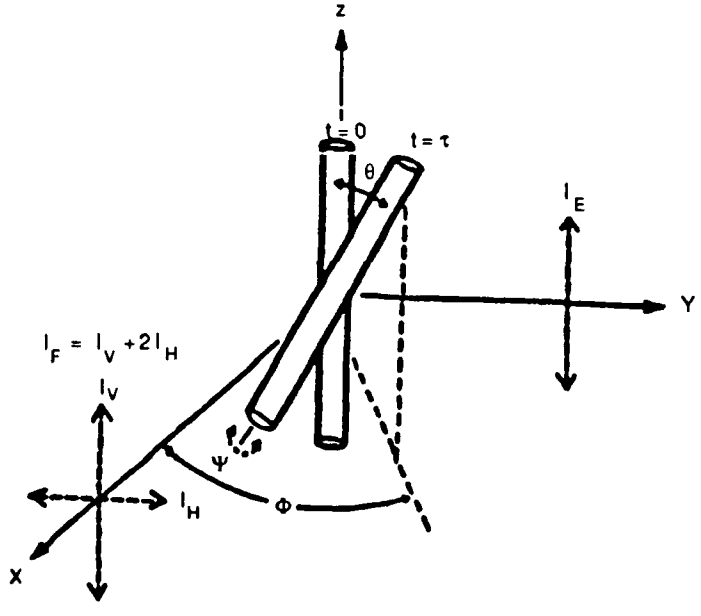


Figure 1-2 Fluorescence anisotropy of a cylindrically-symmetric fluorophore.

When polarized exciting light is directed toward a chromophore molecule whose excitation dipole moment is aligned with the electric vector of the exciting light, the chromophore will preferentially absorb this light. Since the absorption process is so much faster than molecular rotations, the use of oriented exciting light creates a population of preferentially-oriented excited fluorophores. This is referred to as photon-selection. Since the emission of a photon by the excited fluorophore requires a much longer time (the lifetime, τ, of the excited state) than absorption, the fluorophore can often reorient before emission occurs. This is illustrated in Figure 1-2.

If such a situation occurs (i.e., the rotational correlation time of the excited molecule is less than or on the order of excited state lifetime), the emitted photon will no longer be polarized parallel to the exciting photon, even if the molecular excitation and emission dipoles are parallel within the fluorophore. The resulting polarization of fluorescence is often defined in terms of the steady-state fluorescence anisotropy and from Figure 1-2 it is clear that the greater the extent of reorientation of the fluorophore during the lifetime of its excited electronic state, the smaller will be the observed fluorescence anisotropy, to the extent that $r = 0$ for complete fluorophore reorientation ($I_V = I_H$). It is in this sense that fluorescence anisotropy provides a measure of membrane mobility. This is a key point of the proposed method, since it was shown that the genome antibody binding takes place on the membrane surface causing probe's immobilization. It can also be shown that for a rigidly fixed fluorophore

$$r = r_o = 1/5 \left(3 \cos^2 \theta - 1\right) \qquad (1\text{-}2)$$

where the intrinsic anisotropy (r_o) is determined only by the electron distributions of the excited and ground states. From Eq. (1-2) we see that the maximum value for fluorescence anisotropy is 0.4 (for $\theta = 0°$).

During the Phase I research, POC investigated both theoretically and experimentally the advantages and performance of the PFFALP device. As a result of our research, we have identified the optimum excitation light source, a medium to obtain maximum fluorescent quantum yield and an optimal system design, and demonstrated that POC's approach can lead to the successful development of a highly accurate fluorometer to detect the AIDS virus or the AIDS antibody. We have also concluded that our approach has several engineering and molecular design advantages such as the use of incoherent light sources, highly polarization-sensitive detectors and highly specific lipid probes that mimic the natural cell structure [3].

AIDS infection begins as a glycoprotein gp120, on a viral envelope binds tightly to a protein known as the CD4 receptor on the cell surface. The glycoprotein complexes, swept up by the budding virus as it acquires its envelope, are crucial to the HIV's ability to infect new cells. As illustrated in Figure 1-3, a virion or virus particle binds to the outside of the susceptible cell and combines with it, injecting the core proteins and two strands of viral RNA. The virus can then remain latent giving no signs of its presence. Alternatively, it can commandeer cellular mechanism to copy its genes into RNA. The protein and additional RNA are then assembled into new virion that bud from the cells. The process can take place slowly or rapidly.

1.1 Background of POC's Approach

Infection with Human Immunodeficiency Virus (HIV) has been linked to several clinical manifestations, the most severe being Acquired Immune Deficiency Syndrome (AIDS). The need for a reliable test to identify HIV infected individuals and potential blood donors is of paramount importance. In the United States the first of the test kits to identify HIV-infected blood or plasma samples was licensed in March of 1985. The licensed test kits are devised to detect antibodies to HIV antigens in sera and employ the Enzyme-Linked Immunosorbent Assay (ELISA). The inherent disadvantage of ELISA-based tests which make use of partially purified viral lysates is that they are antibody tests and not tests for AIDS. It is thought that about 5% of those in whom the virus is present give a negative antibody test. The other shortcoming of the routinely used tests is their inadequate sensitivity which has led to an increase in the number of the false-negative test results. And, finally, the major problem of the ELISA-based tests lies in the interpretation of the positive results obtained. Due to insufficient specificity of the test, two additional ELISA tests routinely have to be performed on every sample that initially screened positive. If either of these two tests is positive, the blood is classified as repeatedly reactive. For the final confirmation, a different test, called the Western immunobloting, is routinely performed. The latter procedure is more reliable but it is time-consuming, expensive, and requires a highly qualified and experienced operator.

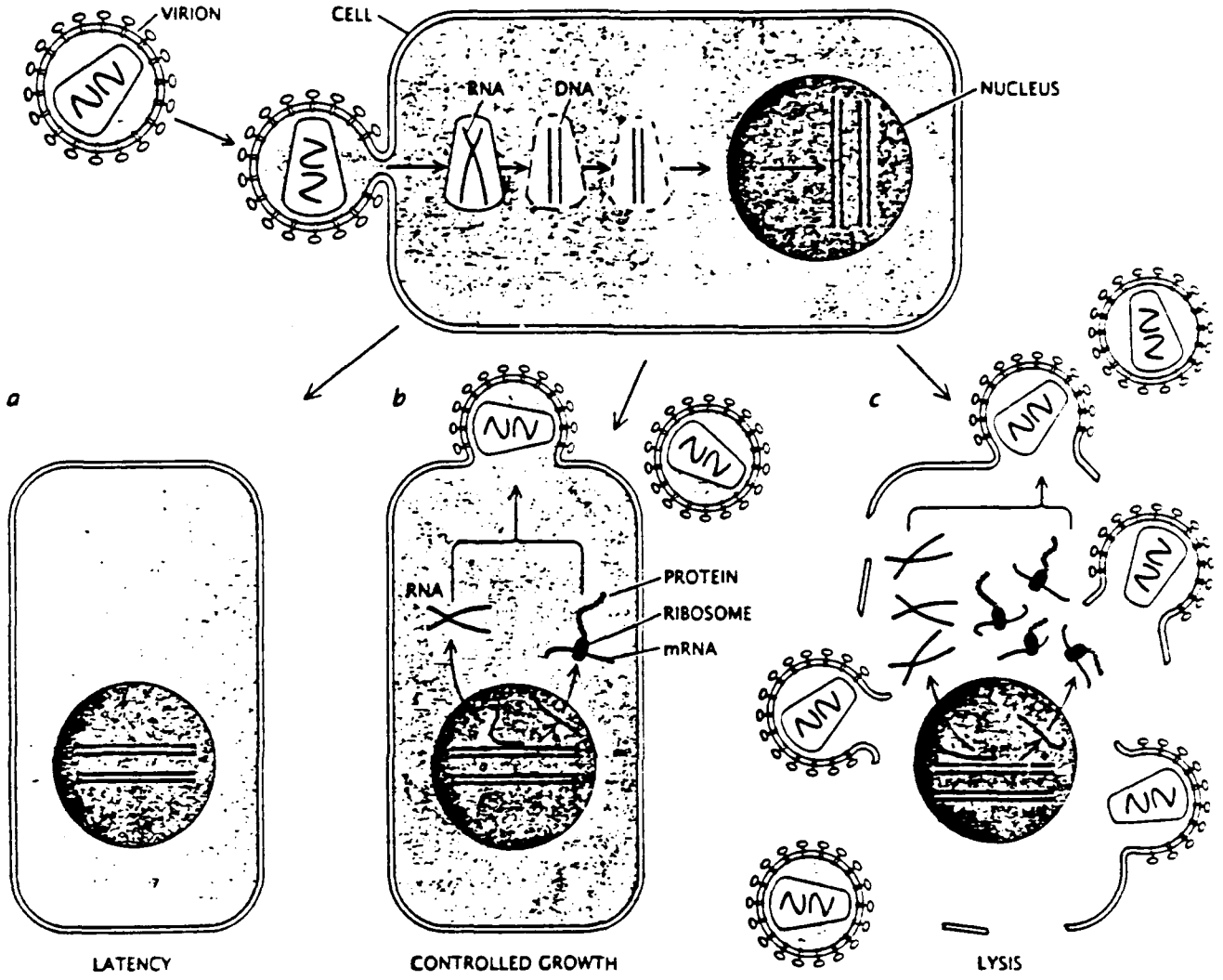


Figure 1-3 Schematic illustration of the AIDS virus infection process, William Haseltine and Flossie Wong Staal, Scientific American, October, 1988.

POC's method is based on binding the ligand to its specific membrane receptor that is accompanied by conformational changes of the receptor protein. This transformation in turn can induce changes in the molecular organization (fluidity and packing) of the lipid environment of the receptor. Changes in the packing characteristics can be followed by incorporating a fluorescent lipid probe into the target membrane. It was previously demonstrated that such probes faithfully mimic the behavior of natural lipids and are able to penetrate into the boundary regions of the membrane proteins [3-5]. The fluorescent labeled lipids in the boundary regions will reflect conformational changes in the receptor protein. Moreover, the primary effect of binding is amplified in this

system, since it is transmitted from one receptor molecule to many lipid molecules. The changes in the packing characteristics of the lipids can be easily monitored in a highly sensitive way by measuring the fluorescence anisotropy of the lipid probes (FALP) embedded in the membrane.

This approach has already been successfully employed in monitoring ligand-receptor interactions in various systems. Monitored interactions include toxins [6], drugs [4], and antibodies [7] binding to their specific receptors. The advantages of the FALP method are following: (i) high sensitivity, surpassing that of the radioligand binding experiments; (ii) specificity of the response, observed only upon high affinity binding of the ligands to the receptors; (iii) enables quantification of the binding interactions, since the measured response is directly proportional to the concentration of the ligand; (iv) time-effective since the maximal response occurs within minutes after addition of the ligand; and (v) provides earlier detection due to higher sensitivity.

POC's new PFFALP will be a significant improvement not only in existing PF applications, but in the application of AIDS virus detection, in particular. The basis of its operation is the fluorescence anisotropy of the lipid probe material. This material emits a polarized fluorescence when illuminated with polarized light. The polarized fluorescence will have a different state of polarization due to the presence or absence of the AIDS virus or AIDS antibody at the lipid probe sites.

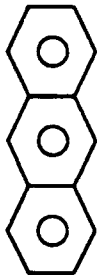
2.0 SUMMARY OF PHASE I RESULTS

In this section of the proposal, we will summarize the Phase I results in meeting our research objectives.

2.1 Phase I Technical Objectives and Achievements

The purpose of the Phase I research was to demonstrate the feasibility of fabricating the PFFALP device that will selectively and accurately detect the AIDS virus using a unique chemical probe in solution form. In order to demonstrate such feasibility, we concurrently pursued three objectives: 1) development and/or acquisition of chemical probe material, 2) assemblage of optical components to construct a PFFALP device, and 3) characterization of probe chemicals and testing of its ability to fluoresce when its molecular environment is changed in the test solution.

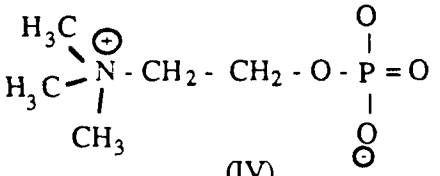
In order to demonstrate the PFFALP device's ability to detect the AIDS virus, we initiated a broad theoretical and experimental study of the chemical synthesis of the probe, processibility and its application into the PFFALP device. This involved the successful development and/or purification of anthrylvinyl labeled: sphingomyelin (ASM), 1-acyl-2 [1,2 (g-anthryl)1 L-transdocanoyl]-sn-glycerol-3-phosphocoline(CAPC)), 1,6-diphenyl-1,3,5-hexatrine, DL-12 (g-anthryl) steric acid and L-alpha-phosphatidylcholine dimyristoyl, probe characterization at different excitation wavelengths and fluorescence efficiencies as well as degree of fluorescent anisotropy in organic solvent environments. As a result, two basic approaches and two probe chemicals have been selected as the most promising for further testing, evaluation and process optimization. The two chemical probes selected are illustrated in Figure 2-1 (a) and (b).



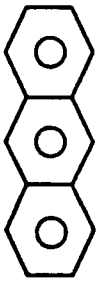
$$CH=CH(CH_2)_9COOH + H_3C(CH_2)_{12}-\underset{H}{\overset{H}{C}}=\underset{H}{\overset{H}{C}}-\underset{OH}{\overset{H}{C}}-\underset{CH_2}{\overset{H}{C}}-NH-\overset{O}{\overset{\|}{C}}-R$$

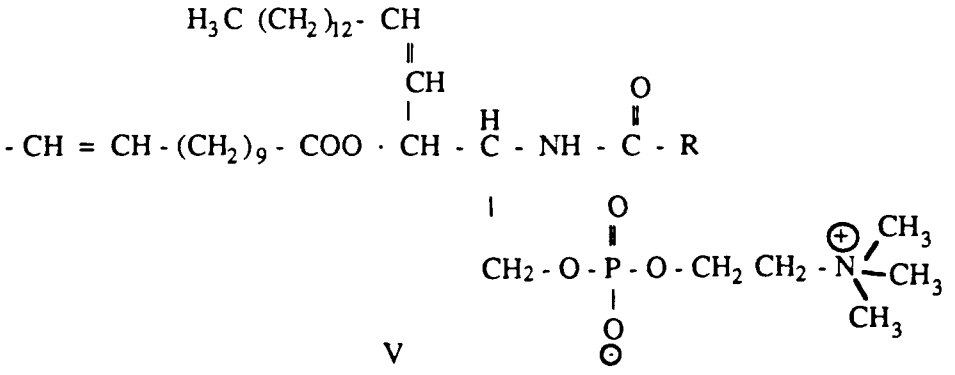
12(9-anthryl)11-dodecenoicacid

(II)



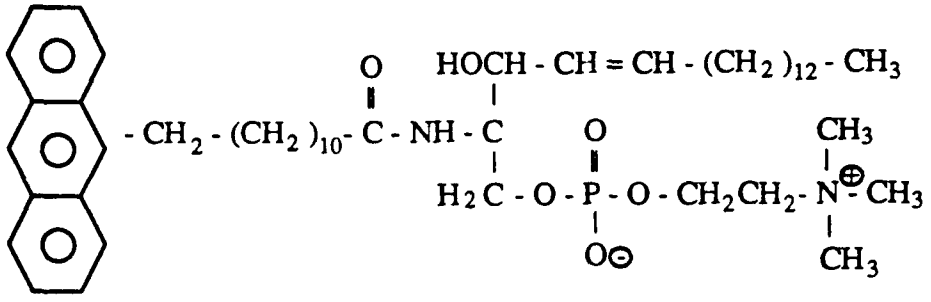
(IV)
Sphingomyelin





V

2-1(a)



(b)

Figure 2-1 (a) N-[1,2-(g-anthryl)-11-trans-dodecenoyl] sphingo-sine-1-phosphocho-line (ASM); (b) 1,2-(g--anthracene) dodecanoyl sphingomelin (ADS).

The synthesis of ASM was accomplished by Prof. Bergelson according to the method described in Ref. [5] whereas CAPC synthesis was accomplish by Sigma Chem. Co. The probe design and development efforts took into account the following important factors: (a) entry of the ASM or CAPC molecules into the lipid bilayer do not alter the orientation and confirmation of polar head groups of the host lipid, (b) anthryl fluorophore moiety is apolar, nonbulky and resides at the loosely packed hydrocarbon chains of the bilayers, (c) such association as in (b) should not create any independent motion in aliphatic regions of ASM or CAPC, and (d) the quantum yield is high and the excited state lifetime of fluorophores is short.

The two basic approaches that could be employed to demonstrate the feasibility of the proposed concept using both ASM and CAPC were studied. The first concept would employ labeling of Burkitt lymphoma EB-3 cell with ASM by dipping the cells in 1:100 (ASM:alcohol) solution and mixing with AIDS genome, antibody, etc., as illustrated in Figure 2-2. The other method would involve simply mixing the probe material separately with healthy serum, AIDS-virus-infected serum, and antibody in alcohol solvent and measuring the fluorescence (see Figure 2-3). ASM probe material has been shown to specifically reside in only apolar regions of bipolar lipid structures.[8] The comparison of fluorescence quantum yield with AIDS virus or antibody in the test solution and without AIDS virus or antibody in the test solution will clearly indicate that POC's PFFALP fluorometer can be a new methodology to diagnose persons infected with AIDS quickly, accurately and cost effectively.

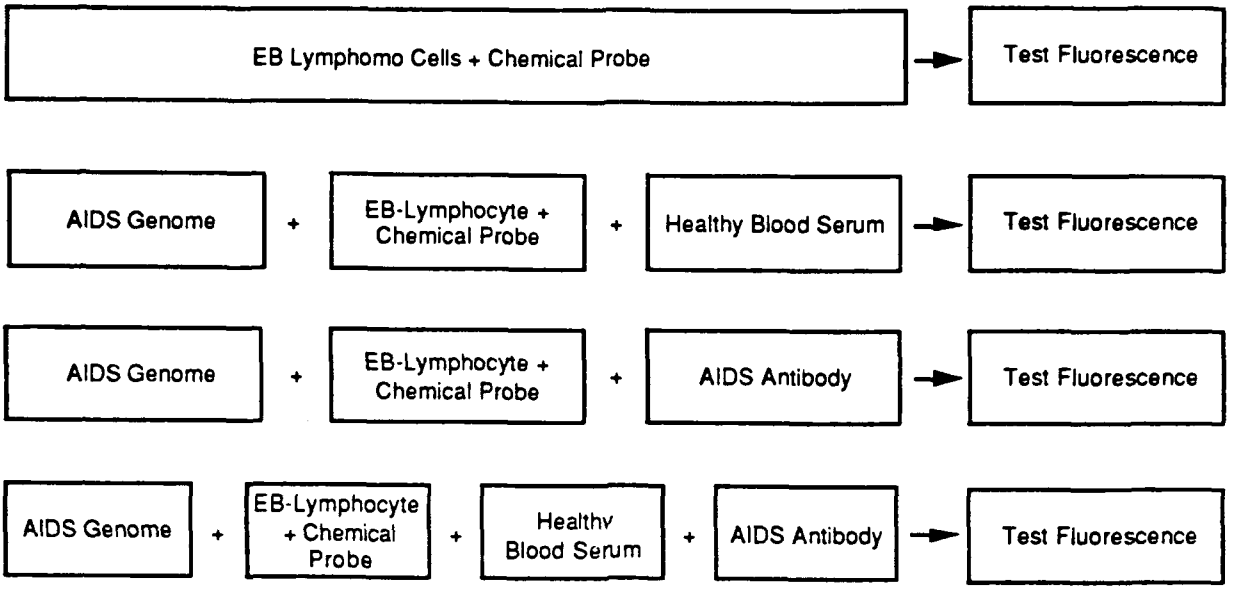


Figure 2-2 AIDS virus detection.

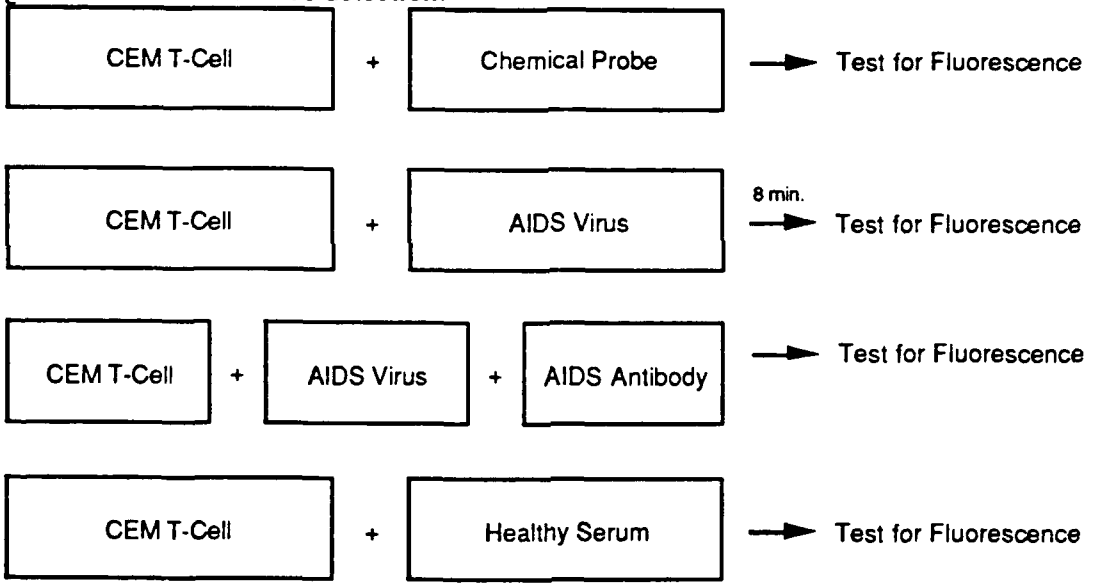


Figure 2-3 AIDS virus/antibody detection test.

2.2 Feasibility of POC's Experimental Demonstration of PFFALP Concept for Peripheral Blood Mononuclear Cells (PBMC)

We have attempted to duplicate studies demonstrating that reaction of an antibody with a cell surface antigen significantly affected the fluorescence of a probe previously inserted into the T-cell membrane. Additionally, in order to assess the possibility that the ASM or CAPC probe might be

used with formalin-fixed human lymphocytes, we have studied the effect of the combination of T-cell surface antigen and antibody on the behavior of a fluorescent probe previously incorporated into the T-cell membrane.

PBMC obtained by Ficoll-Isopaque separation from normal blood donors were washed with phosphate-buffered saline (PBS, 0.14 M NaCl and 0.01 M Na Phosphate pH 7.2). Such PBMC were used fresh or after fixation with 1% formalin for 70 hours at room temperature. After washing two times with phosphate buffer solution, the cells were incubated for 7 minutes with the fluorescent probe ASM or CAPC. Fluorescence assay measurements were taken both before and after washing two times with PBS. The washed cells, 1 ml containing 10^6 PBMC, were then incubated 20 minutes at room temperature with 100 ul of a mouse monoclonal antibody (T101) to the T-cell sheep erythrocyte receptor. The amount of T101 added was sufficient to allow detection of T-cells with fluorescent-labelled anti-mouse immunoglobulin. After incubation for 20 minutes at 30°C, the antibody-coated cells were washed and resuspended in 1.0 ml of PBS and again subjected to fluorescence assay with ASM or CAPC probes.

2.3 Experimental Results

As summarized in Table 2-1, the fluorescence measurements were carried out at Harbor-UCLA Medical Center on October 29, 1990 under the supervision of Dr. Gildon Beall, M.D. The PBMC cells were grown in-house and the samples were prepared as described above. The fluorescence measurements for phosphate buffer solution (PBS) with formaldehyde washings, PBS, methanol 70%, etc., were conducted with parallel and perpendicular polarization. The measurements were continued with inactive T-cells in PBS, T-cells in the presence of 10 µl up to 200 µl probe solution. The fluorescence measurements were further conducted with active T-cells in PBS alone and in the presence of various levels of probe solution. The experimental setup was displayed in Figure 1-1 and a close-up of sample holder setup is depicted in Figure 2-4.

Table 2-1 Results of AIDS Probe Testing at Harbor-UCLA Medical Center, 10/29/90

	Fluorescence Intensity in Amps		
Sample Composition	Polar 90°(↑) ⊥	Polar 0° (↓) ∥	%P
A) Phosphate Buffer Soln. with Formaldehyde Washings	2.6×10^{-6}	$32 \times 10^{-7} \equiv 3.2 \times 10^{-6}$	10.3
B) PBS only	$2.6, 2.6 \times 10^{-6}$	$3.2 \times 10^{-6}, 3.1 \times 10^{-6}$	—
C) MeOH (70%)	16×10^{-7}, 17, 17	20×20^{-7}, 20, 17	5.5
D) Inactive T-Cells in PBS only	18×10^{-7}, 18, 18	17×10^{-7}, 18, 17	1.8
E) #D plus 10 µl of probe solu.*	18×10^{-7}	18×10^{-7}	
F) #D plus 20 µl of probe solu.*	12×10^{-7} stable	over 5 min. period	
G) #D plus 30 µl of probe solu.*	18×10^{-7}	18×10^{-7}	
H) #D plus 100 µl of probe solu.*	32×10^{-7}	31×10^{-7}	1.6
I) #D plus 200 µl of probe solu.= 1 mg	28×10^{-7}	34×10^{-7}	9.6
J) Active T-cells (4×10^6/ml) in PBS + 20 µl of probe solu.*	11×10^{-7}	13×10^{-7}	8.3
K) #J plus 20 µl probe solu.*	13×10^{-7}	15×10^{-7}	7.1
Sample Composition	Polar 90°(↑) ⊥	Polar 0° (↓) ∥	%P
L) #J plus 60 µl probe solu.*	11×10^{-7}, 11	14×10^{-7}, 15	13.7
M) #J plus 120 µl probe solu.*	9×10^{-7}, 10, 10	14×10^{-7}	18.3
N) J plus 1 mg probe	$19 \times 1^{0-7}$	25×10^{-7}	13.6
O) 1 mg probe in 2:1 $CHCl_3$/MeOH Aligned filters + optics	218 x 10-7 no change	229 x 10-7 no change	2.4
P) Changed focus of UV source	400×10^{-7}	400×10^{-7}	
Q) Supernatant of Washed Active T-cells	38×10^{-7}	38×10^{-7}	
R) Supernatant from Inactivated T-cells	30×10^{-7}	34×10^{-7}, 34	6.3
S) Active T-cells after wash	15×10^{-7}, 15	15×10^{-7}	0
T) Inactivated T-cells after wash	23×10^{-7}	23×10^{-7}	
U) 1% Formaldehyde in PBS	5 x 10-7	5 x 10-7	

V) #U plus 10 µl probe solu.*	14×10^{-7}	14×10^{-7}	
W) #U plus 20 µl probe solu.*	26×10^{-7}	26×10^{-7}	
X) PBS + 200 µl probe solu.*	14×10^{-7}	15×10^{-7}	3.4
Y) Actives cells (#S) plus antibody	6×10^{-7}	6×10^{-7}	0
Z) Inactivated T-cells + antibody	15×10^{-7}	17×10^{-7}	6.3
$\%P = \frac{I_{11} - I_{\perp}}{I_{11} + I_{\perp}}$			

NOTES: All samples were 1 mL, measured in 1 cm x 1 cm cuvette.
* Probe solu. = 1 mg dissolved in 0.2 mL of MeOH (70%)

Low levels of fluorescence of probe in T-cell/Buffer solutions (samples D → N) was observed for active and formaldehyde inactivated T-cells. This suggests either competitive light absorption by the T-cells or PBS, or quenching of the probe excited state by T-cells or PBS. When the probe was included in PBS only (sample X) a low level of fluorescence, approximately one-tenth of the fluorescence intensity of probe in solvent alone (sample O) was observed. This indicates that PBS is responsible for probe quenching or competitive light absorption. Because these fluorescence intensities are low (samples A, B, C) , the probe concentration needs to be increased to enhance the test sensitivity in future tests. To improve this, we would need to try either using different buffer solutions(s), or increased probe concentrations. In future experiments, the optimization of optics and T-cell antibody interaction should be carried out to increase sensitivity.

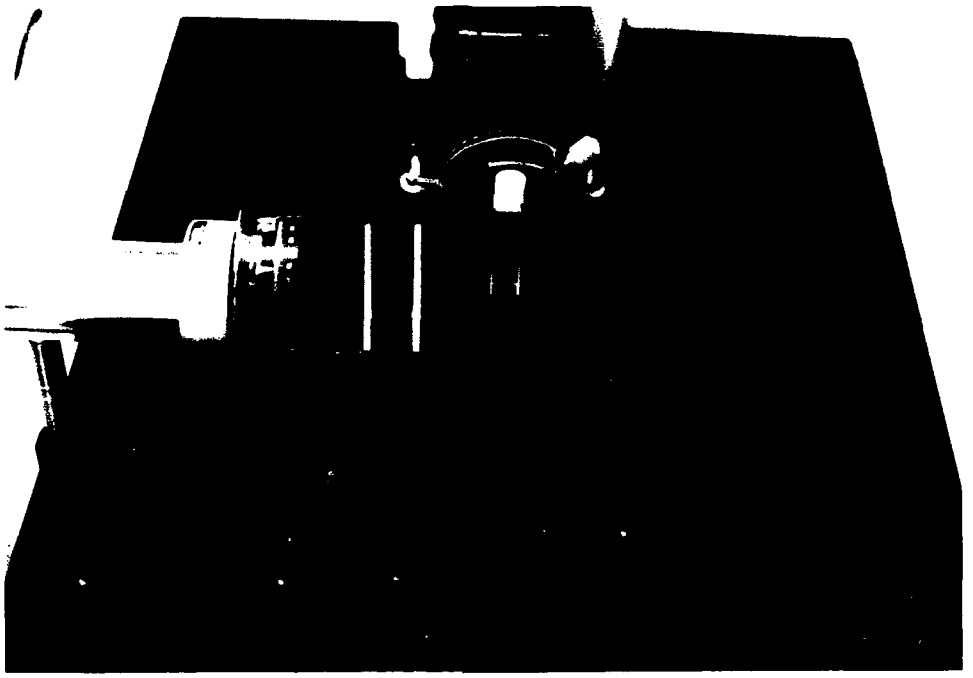


Figure 2-4 A close-up photograph of a sample holder setup which is a part of the PFFALP device.

Conclusions:

The tests do show the probe is able to detect changes in cell environment for active or formalin inactivated T-cells. Addition of antibodies causes a detectable change in fluorescence intensity.

2.4 Characterization of the Fluorescent Probe

To characterize the FALP materials and find the degree to which they florescence anisotropically the fluorometer components were assembled as shown in Figure 4-9 of Section 4. The excitation wavelength is 365 nm. Fluorescence peaks at around 420 nm. Four FALP materials were tested. They are 12 (9 anthracene dodecanoyl-sphingomyelin (ads), 1,6-diphenyl1, 3, 5 - hexatriene (dph), 1,2-(9-anthroyl) stearic acid (asa), L-α Lecithin sphingomyelin (LSM), and L-α phosphatidyl choline, dimyristoyl (pdd). We generated sufficient data for $I_{\|}$ and $I_{\perp}$, which are the intensities of fluorescences through the vertical and horizontal analyzers respectively, for each of the above probes. Apparently, they emit a rather uniformly unpolarized fluorescence. Without the presence of the biological material, the polarization value (P) is very small. A change in the P value, when the biological material is added, confirms the success of the FALP in detecting the biological material.

Table 2-2 shows a sample of data taken on the four FALPs. The values of $I_{\perp}$ and $I_{\parallel}$ do not differ for each pair of readings, suggesting very little fluorescence anisotropy. It is expected that $I_{\perp}$ and $I_{\parallel}$ became different when the biological material was introduced with the FALP material. Figure 2-5 is a photograph illustrating the experiment showing no fluorescence with the solvent system, whereas Figure 2-6 demonstrates a strong fluorescence when a probe is introduced into the solvent.

Table 2-2 Study of the states of polarization of fluorescence from four FALP candidates

			(Anthryl) Stearic Acid		Diphenyl-Hexatrine		(Anthracene)-Dodecanoyl		Phosphatidycholine,dimyristoyl	
Polarizer Angle	Analyzer Angle A_1	A_2	$I_{\perp}$ (μw)	$I_{\parallel}$ (μw)	$I_{\perp}$ (μw)	$I_{\parallel}$ (μw)	$I_{\perp}$ (μw)	$I_{\parallel}$ (μw)	$I_{\perp}$ (μw)	$I_{\parallel}$ (μw)
0	45	-45	2.20	2.13	0.360	0.320	0.196	0.190	0.013	0.013
45	45	-45	1.42	1.42	0.260	0.250	0.117	0.116	0.011	0.011
90	45	-45	1.46	1.46	0.260	0.270	0.127	0.124	0.010	0.011
0	90	0	2.10	2.10	0.340	0.350	0.181	0.180	0.012	0.013
45	90	0	1.41	1.41	0.280	0.280	0.115	0.113	0.010	0.010
90	90	0	1.43	1.43	0.083	0.091	0.127	0.125	0.011	0.010

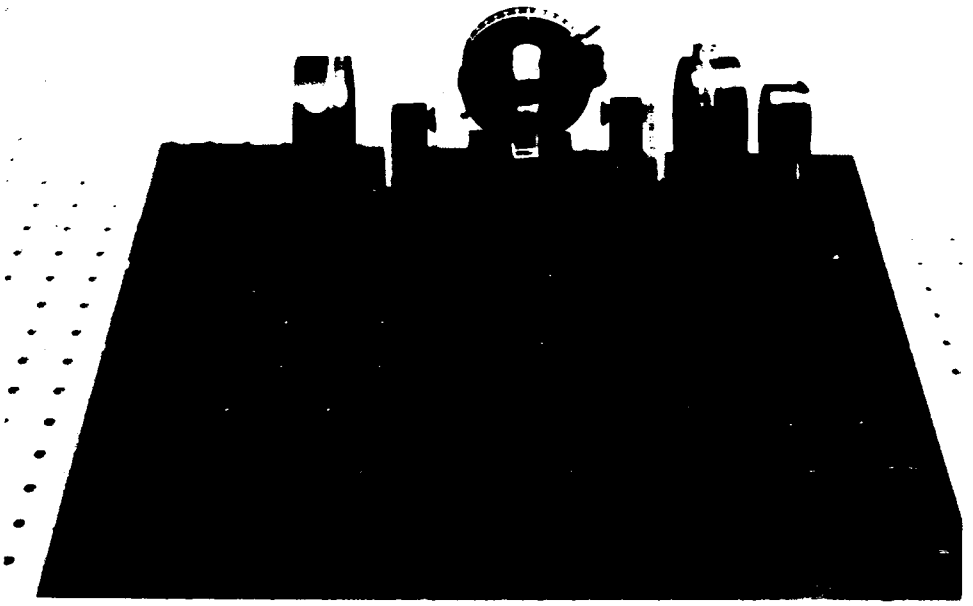


Figure 2-5 Photograph of the experimental setup demonstrating that there is no fluorescence with the solvent system.

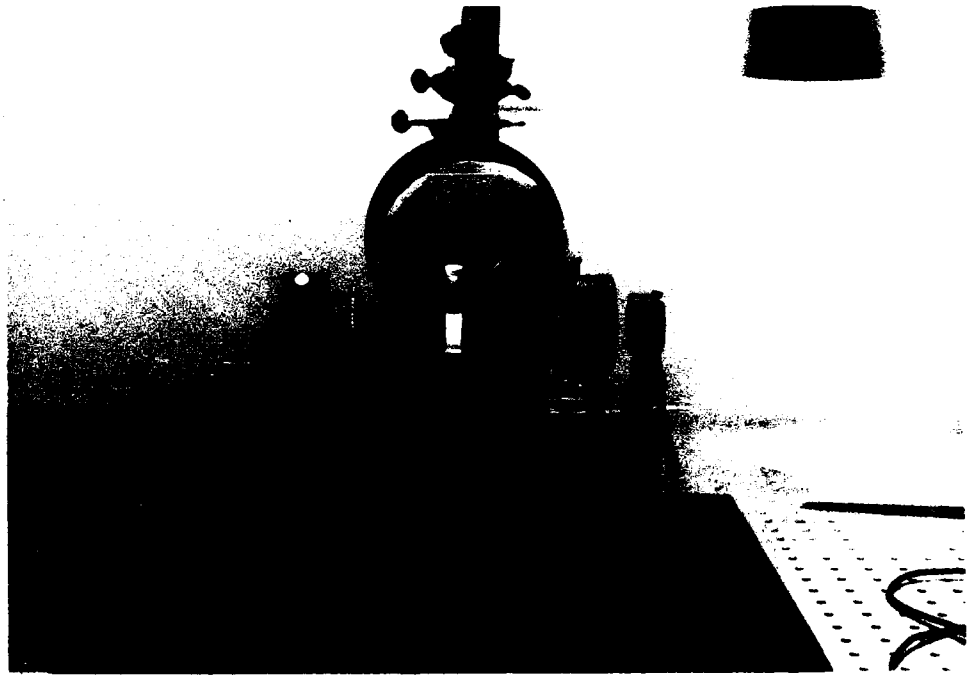


Figure 2-6 Photograph of the experimental setup demonstrating strong fluorescence when the probe is present in the solvent system.

3.0 TECHNICAL DISCUSSION

The routine immunodiagnostic tests involve the detection of antibodies to a given antigen developed as a result of immunization or in consequence of infection. In a typical test, blood is drawn from a patient with an infection and serum is tested against a know antigen. The classical serological tests utilize techniques such as precipitation (ring and capillary tube test), agglutination, toxin-antitoxin tests, virus neutralization, immunocytolysis and complement fixation. The more recent immunodiagnostic techniques are based on: (i) separating different antigens that may be present in antigen material or serum by using support media such as agar gel and polymeric membranes through diffusion, osmosis, electrical charge or combinations of these; (ii) attaching soluble antigens or antibodies to cells (red blood cells) or to latex particles that result in a visible reaction such as lysis or agglutination when antigen antibody unite; and (iii) by labeling immunoglobulins or antigens with dyes that fluoresce upon UV irradiation, radioactive materials that are detected by radioactive sensing devices, electron dense materials that display uniform spheres in electron microscopes and enzymes whose presence is detected by adding proper substrates to the precipitation. The principle of immunofluorescence is depicted in Figure 3-1, whereas diagnostic techniques are summarized in Table 3-1.

The direct detection of the HIV virus (see Figure 3-2) is made possible due to the knowledge of a specific interaction occurring early in the HIV infection: namely, the specific binding of the virus through one of its envelope glycoproteins to a cellular glycoprotein CD4. The CD4 antigen is expressed on the surface of human lymphocytes; its specific binding to the HIV virions is well characterized and the CD4 binding (recognition) site has been defined. Antibodies to HIV could be detected through their specific interaction with the known components of the viral envelope including the protein gp41.

The binding interactions (see Figure 3-3) between CD4 and serum HIV or between gp41 and serum anti-HIV can be registered by the ultrasensitive Fluorescence Anisotropy of Liquid Probes (FALP) method. This requires: (a) preparation of small lipid vesicles which contain a fluorescent lipid probe such as anthryvinyl-labelled sphingomyelin (5) and peptides encompassing the binding site of CD4 or gp41; and (b) construction of a specially designed fluorescent pclarimeter. Technically, the test consists of incubation of the lipid vesicles with the sera and measurements of the change in the steady-state level of the fluorescent anisotropy (r-value) of the lipid probe. Binding of the serum HIV virions to the CD4-specific peptide or binding of the anti-HIV antibodies to the gp41 carrying vesicles results in the change of the r values due to changes in fluidity and packing density as illustrated in Figure 3-4.

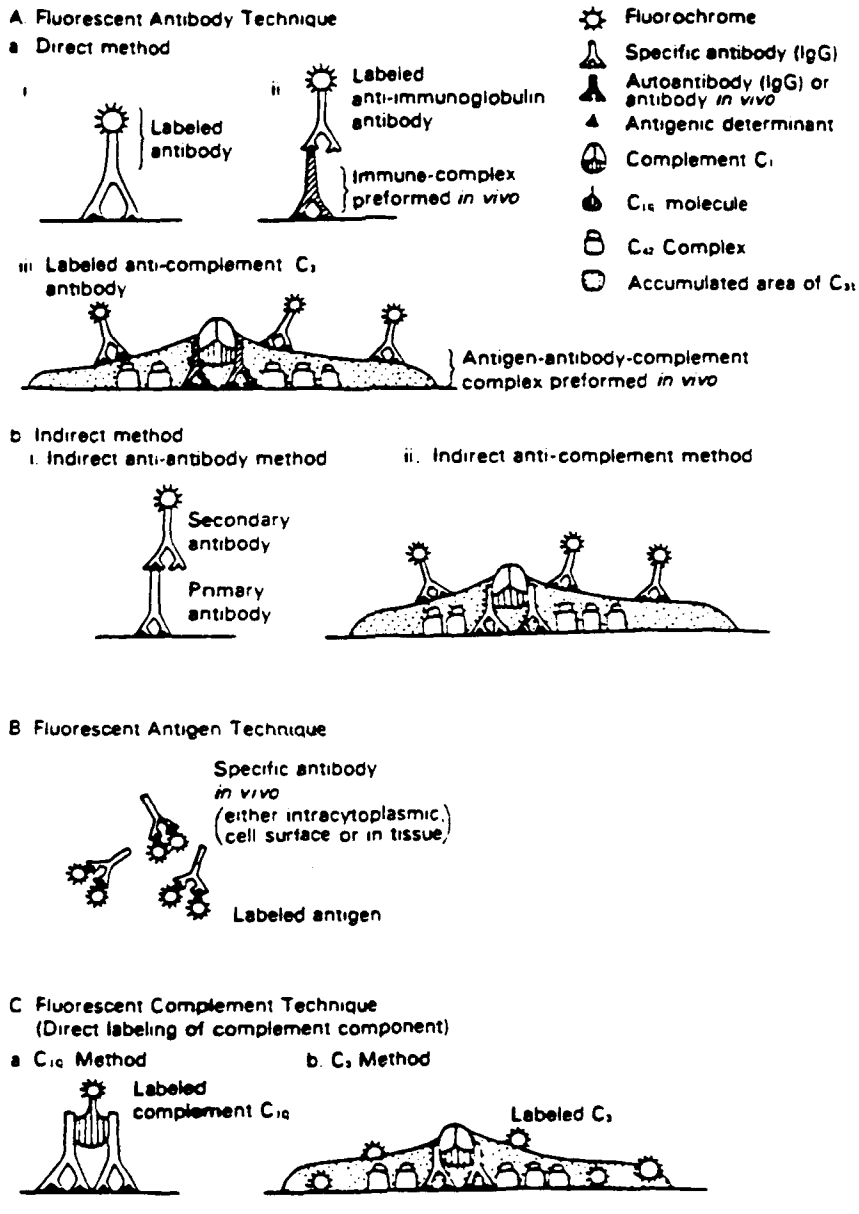


Figure 3-1 The principle of immunofluorescence (Basics of Immunology by W. M. G. Amos, Butterworth, Boston, 1981).

Table 3-1 Diagnostic Techniques for HIV-1

Method	Initial Use	Description	Current Status (1988-1989)	Reference Cited
Isolation & culture of HIV-1	1983	Growth & detection of virus in living cells	Clinical diagnosis	[9, 10]
Antibody detection ELISA using cell culture antigen	1985	Detection of antibody	Routine blood screening and clinical diagnosis	[11]
Western blot	1985	Confirmation of antibody detected	Routine confirmation of ELISA	[12, 13]
Immunofluorescence, hemagglutination, radioimmunoprecipitation, and so on	1985	Detection of antibody	Limited Use	[14 -20]
ELISA using recombinant antigen or synthetic peptide	1986	Detection of antibody	Research	[21, 22]
Antigen detection	1986	Detection of viral antigen	Research	[23 -26]

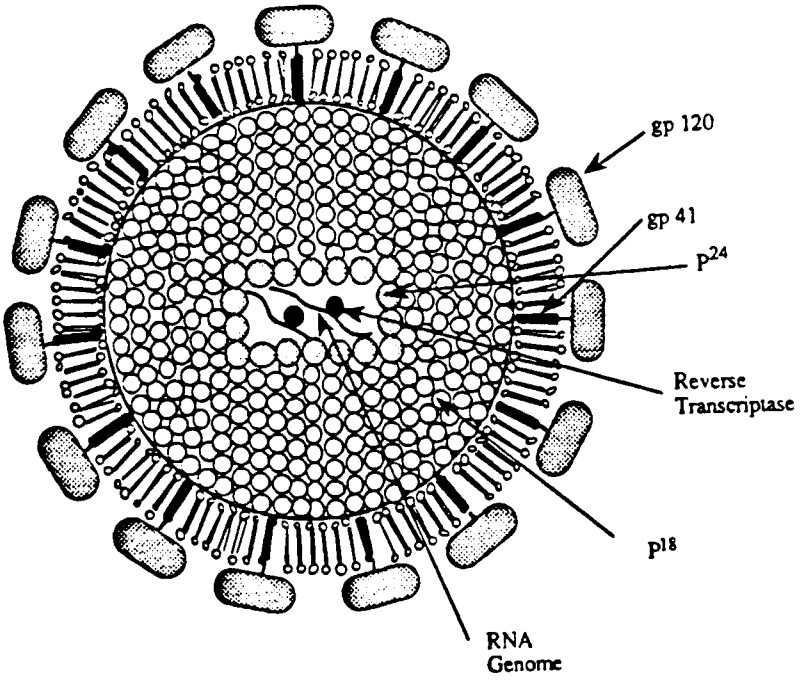


Figure 3-2 Schematic Illustration of Human Immunodeficiency Virus (HIV) from "Biochemistry" by Lubert Stryer (W. H. Freeman & Co. N.Y.).

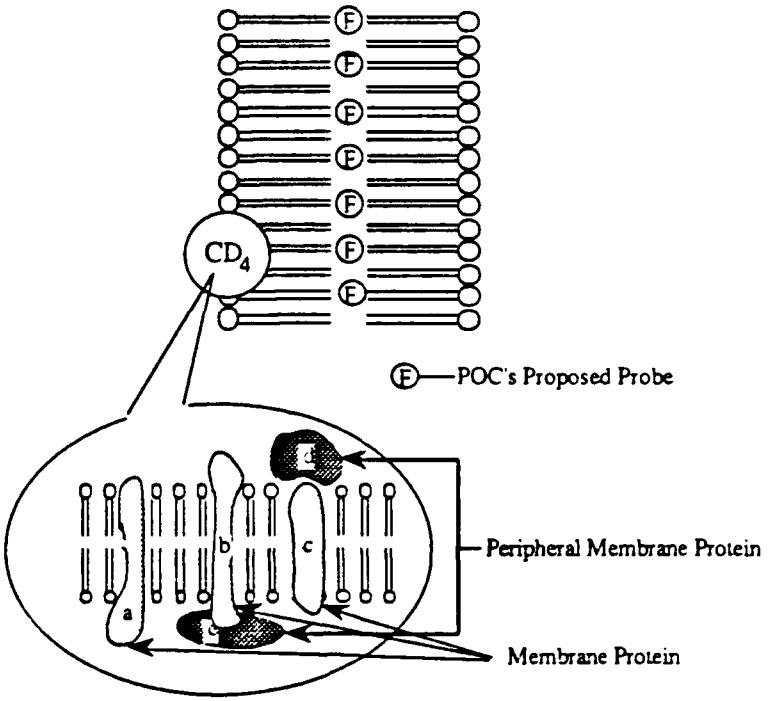


Figure 3-3 Illustration of the HIV infection on a microlevel (figure not to scale).

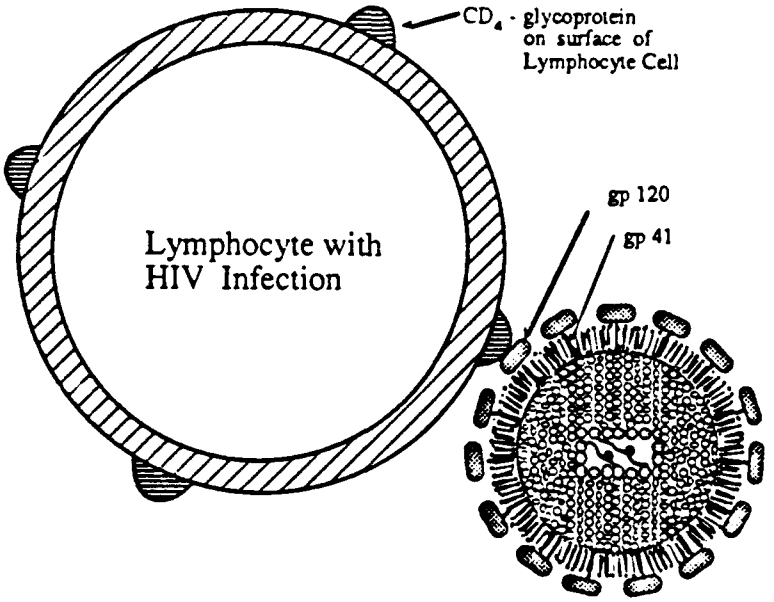


Figure 3-4 Illustration of how CD4 binding to membrane protein affects packing density and/or fluidity within the lipid bilayer.

Preliminary experiments employing the FALP method in HIV detection demonstrated their applicability. When an ELISA-tested panel of sera was used, the FALP technique responded positively to most serum samples which were HIV-exposed by ELISA criteria, while ELISA-negative sera did not induce change of fluorescence anisotropy. The change in r value was concentration dependent, permitting the use of the changes in r produced by a given serum to evaluate the HIV concentration or the antibody level. The critical factors in FALP-based testing are: size and composition of the test vesicles, the nature of the fluorescent lipid probes, the structure of the CD4 or gp41 epitopes and the construction of the polarization fluorometer.

The Polarization Fluorometer for Fluorescence Anisotropy of Lipid Probes is built around the current models for polarization fluorometers, but it is unique in its use of holographic technology to produce a small portable device with all the operational parameters "built-in", allowing accurate readings to be taken by technicians with minimum training. The detection system works by measuring the difference between the state of polarization of fluorescence from "activated" lipid probes and "unactivated" lipid probes. Activated lipid probes imply the presence of the AIDS virus. The fluorescent anisotropic lipid probes emit polarized light, the state of which varies according to the presence of AIDS virus.

3.1 Measurements of the Polarization Anisotropy of Fluorescence

Polarization anisotropy of fluorescence is defined as [27]:

$$r(t) = \frac{(i_{\parallel}(t) - i_{\perp}(t))}{(i_{\parallel}(t) + 2i_{\perp}(t))} \qquad (3\text{-}1)$$

where $i_{\parallel}(t)$ and $i_{\perp}(t)$ denote the intensities at time t of the fluorescence components polarized parallel and perpendicular, respectively, to the plane-polarized pulse of the fluorescence-exciting light. The anisotropy depends on the rotational movements of the fluorescent molecules; in the case of rotational diffusion the relations between r and the diffusion coefficient D_i of a molecule are given by Chuang and Eisenthal [28]. In the special case of a rotating sphere, the anisotropy takes the form:

$$r(t) = r(0)e^{-6Dt} \qquad (3\text{-}2)$$

whereas decays with more than one exponential indicate deviations from the spherical form. Other theoretical aspects and applications of the anisotropy measurements have been reported and reviewed [2]; the aim of this subsection is to discuss some principles and effects to be kept in mind in the construction and use of an apparatus for the measurement of fluorescence polarization.

The actual measurement deals with small difference $\delta(t) = i_{\parallel}(t) - i_{\perp}(t)$, hence pulse light illumination can be considered to increase SNR. However, it is not linear with respect to the integral intensity.

The measured quantity, the probability density $p_1(t)$ for the release of the first photoelectron or (if one uses a photomultiplier with single electron resolution), the probability density $S_L(t)$ for the detection of one and only one photoelectron at time t is related to the fluorescence intensity i(t) through

$$p_1(t) = \alpha q i(t) \left(1 - \int_0^1 p_1(t')\, dt' \right) = \text{constant} \times i(t) \tag{3-3}$$

$$S_L(t) = \alpha q i(t) e^{-n} = \text{constant} \times i(t) \tag{3-4}$$

$$n = \alpha q \int_0^{\infty} i(t')\, dt' \tag{3-5}$$

where α is the quantum efficiency of the photomultiplier, q is the attenuation factor and n is the mean number of photoelectrons released per exciting light flash.

The integrated intensity $\int_0^t i(t)$ is proportional to n, and n can be calculated from the measured detection probabilities w(n) or $w_s(n)$:

$$w(n) = \int_0^{\infty} p_1(t)\, dt = 1 - e^{-n} \tag{3-6}$$

$$w_s(n) = \int_0^{\infty} S(t)\, dt = ne^{-n} \tag{3-7}$$

Both Eqs. (3-6) and (3-7) show the nonlinearity: the detection probabilities are not proportional to the integral value n of the light intensity. The main conclusion that can be drawn from this nonlinearity is the preference for the use of non-single-electron detection, despite seeming advantage of increased sensitivity.

Another point to consider is that using Eq. (3-1) requires implicitly the stability of the apparatus in terms of excitation intensity and detection sensitivity. However, we must be aware of appreciable variations (up to 5%) of the excitation intensity due to fluctuations of the probe concentration in mixture, as well as due to light source instability. The device stability requirements can be met by (i) a suitable optical arrangement including arc lamp current stabilization and (ii) constant stirring of suspension to provide uniform distribution of reacting agents and probe. Another way to avoid the problem is to provide simultaneous measurement of both polarizations so only the relative excitation intensity would be involved in polarization anisotropy calculation:

$$r(t) = \frac{1 - i_{\perp}(t) / i_{\parallel}(t)}{1 - 2i_{\perp}(t) / i_{\parallel}(t)} \qquad (3\text{-}8)$$

Moreover, taking a number of measurements during a sufficiently long time interval and averaging we can make the results insensitive to short time fluctuations.

3.2 Studies In Optical Design and Measuring Technique

There are different fluorometers available to provide the measurement of the fluorescent spectrum and in some cases the fluorescence decay. A typical example of a spectral fluorometer is shown in Figure 3-5. The device represented in the schematic diagram is made by Hitachi as its F-2000 model of Fluorescence Spectrophotometer. (Here we should also mention the LS-100 fluorescence system by Photon Technology Inc.) The most valuable and, thus, expensive feature of a commercially available fluorescence spectrophotometer is a tandem of monochromators put in to isolate the excitation light coming from the xenon arc lamp from the emitted light. Commonly, monochromators are integrated with the wave control digital system. Being very useful for laboratory research and actually supporting numerous discoveries regarding ligand-receptor interactions such devices are too bulky and expensive for routine diagnostic tests. Also, standard spectrofluorometer usually requires highly skilled professionals to operate.

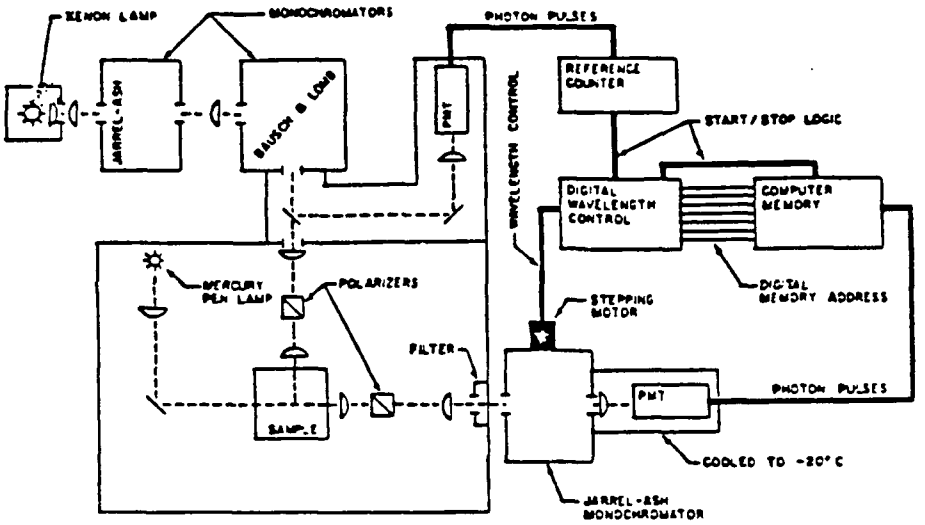


Figure 3-5 Block diagram of a spectral fluorometer.

The feasibility of this design has been shown during Phase I of this project. We have studied this design from the size reduction point of view. The possibility of using a single wavelength (or a narrow spectral interval) for the probe excitation as well as for probe emittance registration follows from the fact that the excitation spectrum of the probe is quite narrow and can be fully represented by, say, a 10 nm spectral interval around the peak, Figure 3-6, achievable by an interferometric filter. At the same time, since the fluorescent emitted spectrum is quite broad with a maximum shifted to longer wavelengths (Figure 3-7), it can be represented by its integrated characteristic. Without losing accuracy, we can measure the total amount of energy emitted by the probe. The only precaution is that the interval of the excitation should be separated from the interval of measurement. This can be easily done by using a UV cutting filter (edge absorbtion filter). Furthermore, the integration over the remaining part of the spectrum increases the sensitivity of the method.

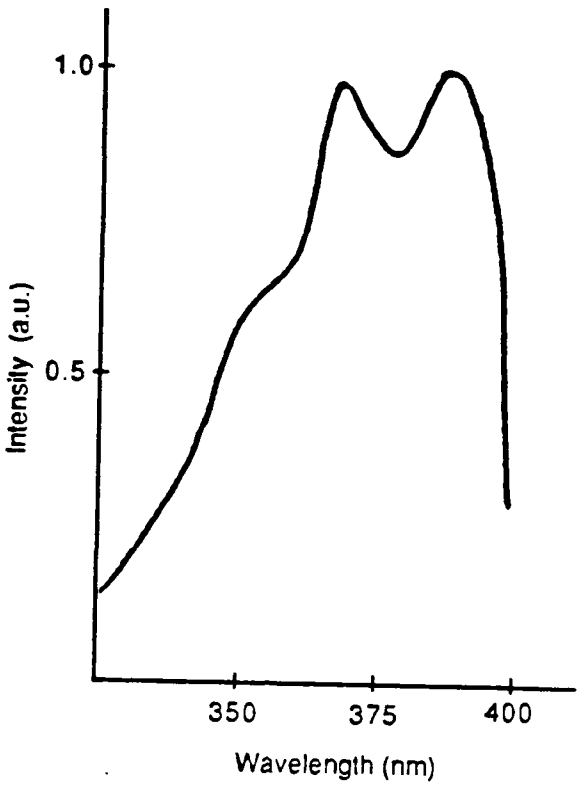


Figure 3-6 Excitation spectrum of the probe.

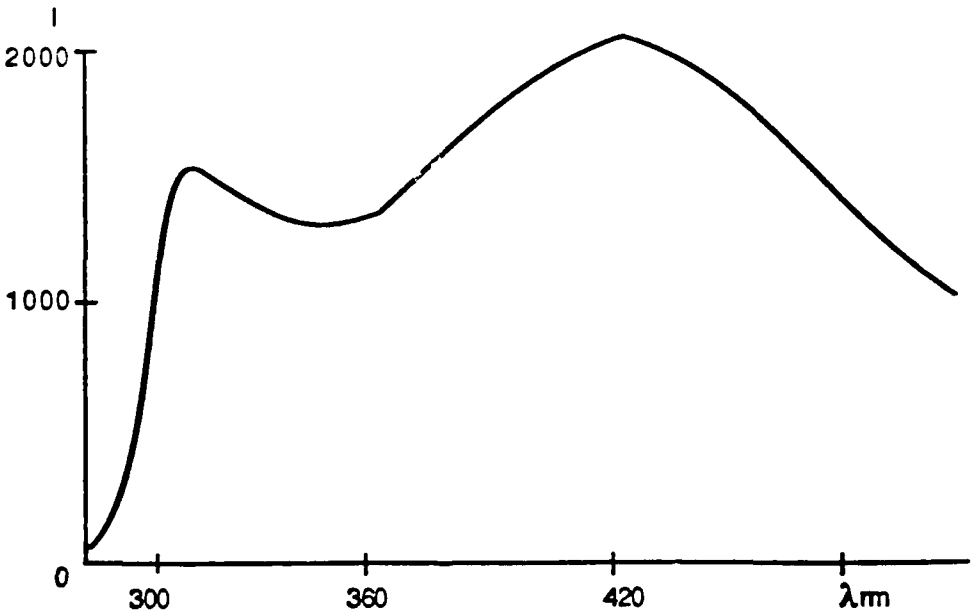


Figure 3-7 The probe emittance spectrum.

The following design was studied to verify the feasibility of the test. An experimental optical setup is shown in Figure 3-8. Although the polarization measurement in an L-format configuration (one photodetector receiving light in the direction perpendicular to the exciting light) has an advantage in suppressing the background due to scattered light or undesired luminescence, it requires four orientations for the polarizers and a significant amount of time to obtain the polarization spectrum. The relatively long period of measurement is especially disadvantageous for polarization measurements of phosphorescence with long lifetimes. In the T-format optical configuration, the emission is viewed simultaneously in both emission directions normal to the direction of the exciting light. The T-format polarization measurement requires only two orientations of the polarizers. We made our design adjustable to both configurations and since the purchase of two photomultipliers is beyond the Phase I budget the present results were obtained in the L-format.

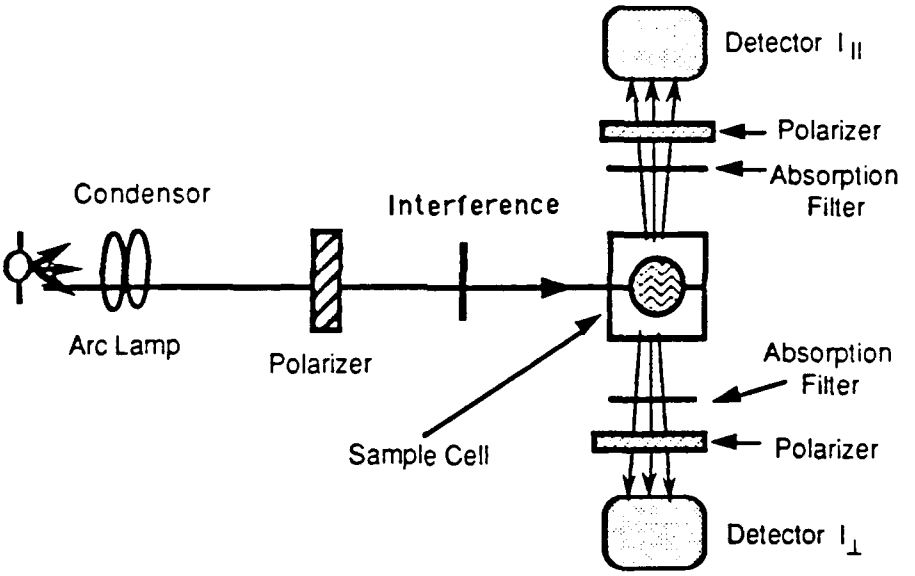


Figure 3-8 Illustration of the experimental setup used for characterizing probe fluorescence.

1. *Light Source*

The following parameters were taken into account when choosing the light source for our polarization fluorometer:

- spectral distribution
- spectral radiance
- total output
- source size and shape

- source stability

An Argon laser seems to be highly suitable as providing already polarized, high power beam in the UV region of interest. POC tested the usage of the Argon laser and found it to be a convenient light source for laboratory adjustment and testing. However, employing an Argon laser in a prototype device is impossible due to the large size of the laser. The best candidate for the light source conforming with all listed requirements is an Mercury (Xenon) Arc Lamp (Figure 3-9). Its spectral distribution contains a very strong peak at 370 nm which corresponds to the excitation spectrum of our probe (Figure 3-10). Cut by a narrow-band filter, the 75 W Hg(Xe) Lamp radiates enough power to cause fluorescence. The mercury arc lamp contains exactly measured amounts of mercury and xenon which acts as a starter gas as the mercury gets vaporized. When the lamps operate the pressure reaches up to 75 bar. This requires a five to ten minute transition period so the lamp runs at higher than normal current and the anode must be at the bottom to ensure proper vaporization of the mercury. It is desirable to have a portion of the bulb covered by a reflecting coating. This speeds up the transition phase and improves the thermal distribution. The possible disadvantage of this type of lamp is that since the bulb temperature influences the mercury pressure, these lamps are sensitive to airflow around the bulb. This effect can be significantly diminished by using a special lamp housing system.

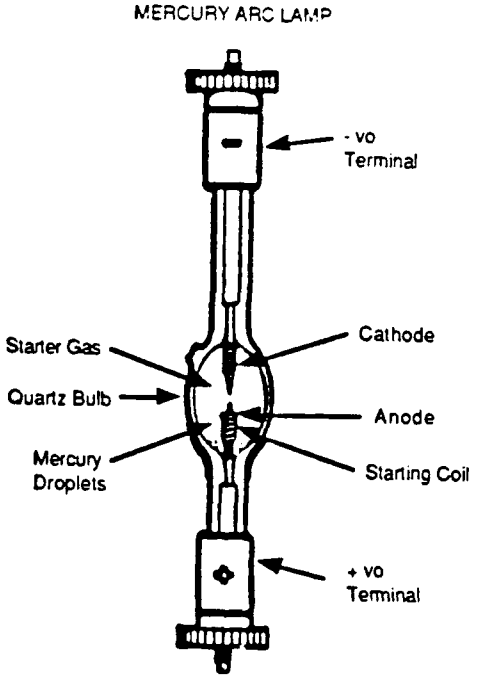


Figure 3-9 Short mercury arc lamp used in POC's experimental setup.

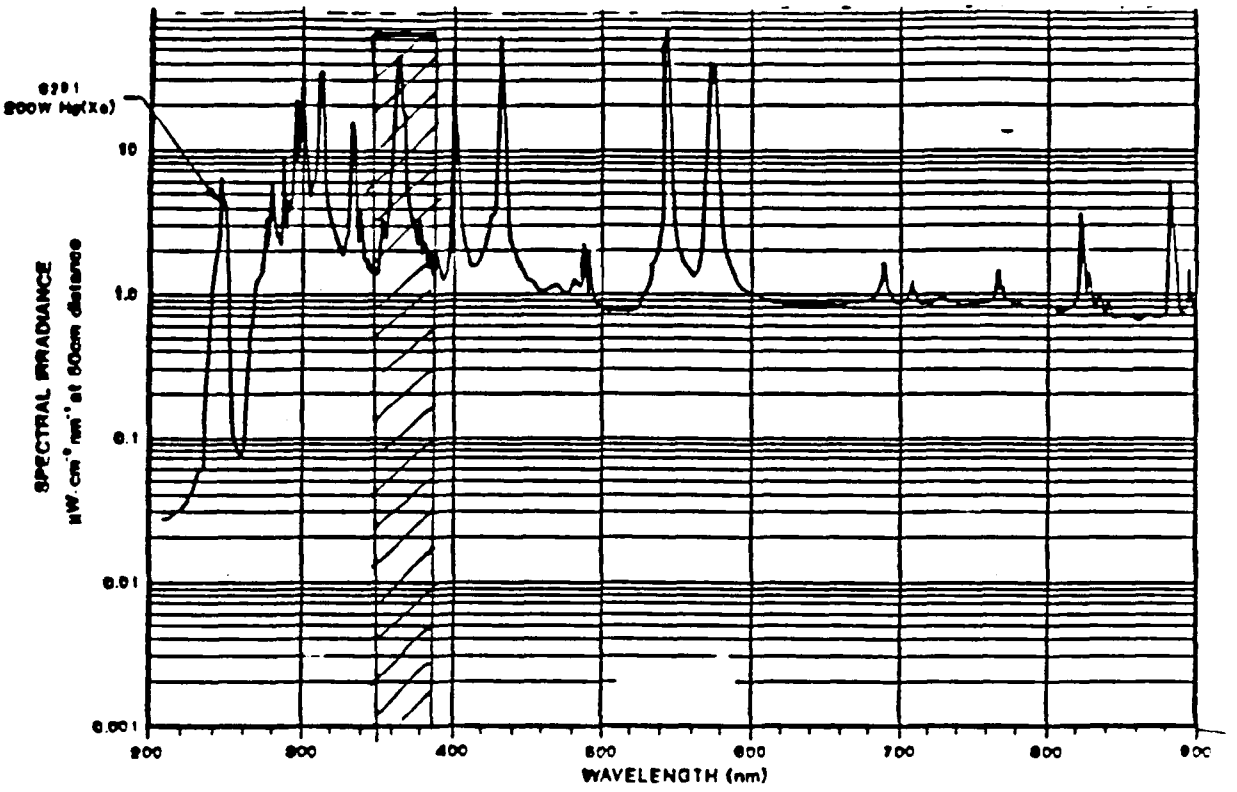


Figure 3-10 Spectral characteristic of mercury arc lamp with filter passed spectral band (shaded).

2. *Narrow-Band Filter*

Narrow-band interference filters permit selection of wavelength intervals down to a few nanometers in width without the use of dispersion elements such as prisms or gratings. They operate on the same principle as the Fabry-Perot interferometer and thus have a limitation on their angular acceptance. To avoid passing of undesired wavelength, the following condition must be satisfied.

$$\cos\theta < \frac{\lambda}{t_{opt}} \qquad (3\text{-}9)$$

where t_{opt} is an optical thickness of the interference filter and θ is the half angular width of the source. Thus, the device geometry could be limited by the restriction of Eq. (3-9). We used a Melles Griot interference filter with wavelength peak centered at 365 nm and a band-pass of about ± 5 nm. Although the peak transmittance of this filter is 25%, it provides enough power for

fluorescence excitation since we collect the light onto a small area of a sample. The spectral characteristic of the Melles Griot band-pass filter is shown in Figure 3-11. It demonstrates a good overlapping with the probe excitation spectrum.

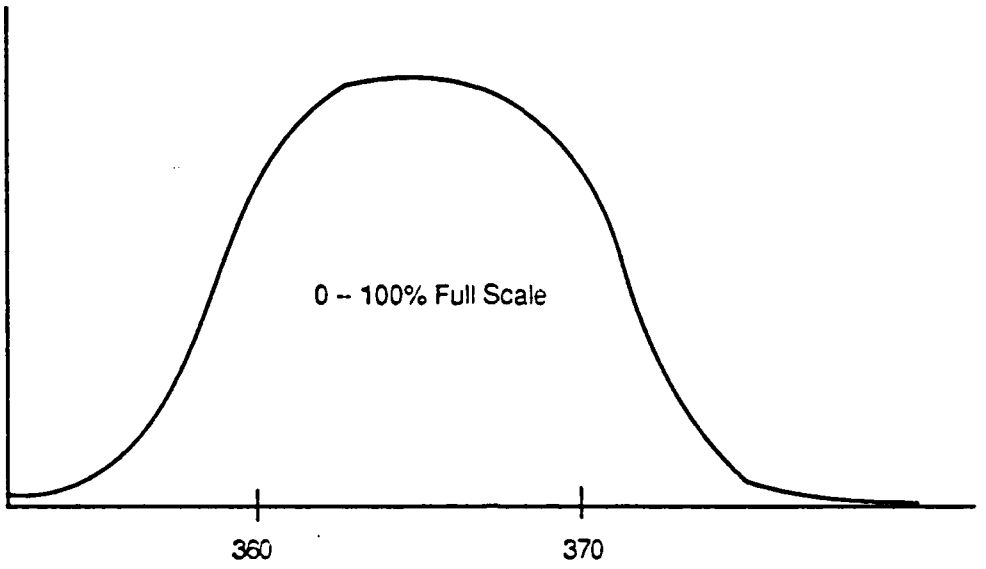


Figure 3-11 Spectral transmittance of the Melles Griot interferometric filter.

3. *Sharp Cut Off Filter*

As was discussed earlier, it is possible for our purpose to measure fluorescence emitted light integrated over spectrum, so only an edge absorbtion filter is necessary. The Melles Griot Colorless Sharp Cut Off Glass Filter has been chosen with a threshold wavelength ~ 390 nm. The maximum transmittance spectral interval of this filter perfectly covers the emitted spectrum of the probe. The spectral transmission curve of the filter verifies this statement (Figure 3-12).

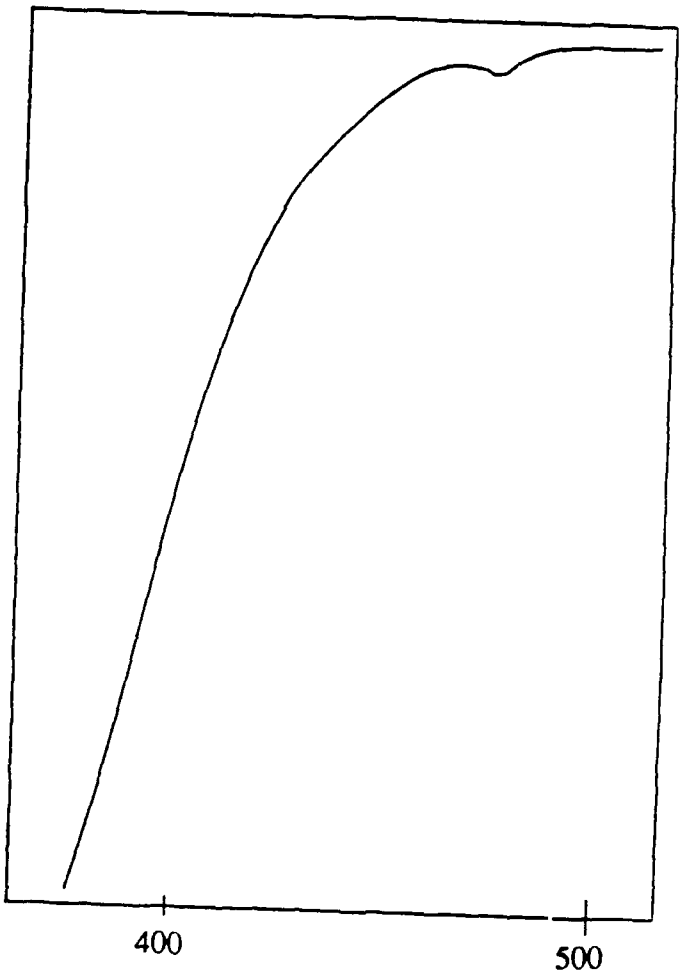


Figure 3-12 Spectral transmittance of the cut-off filter.

4. *Polarizers*

The measurement of the anisotropy of polarization requires a careful adjustment of polarizers. Our main concern is to provide a good separation between two perpendicular directions of measurement. We found that inexpensive plastic dichroic polarizing sheet sandwiched between selected strain free glass plates manufactured by Melles Griot is fully acceptable and provides enough accuracy for our measurement. Advantages of these polarizers include large apertures and acceptance angles, excellent extinction ratio and simplicity in mounting. The transmittance T of a single shut polarizer in a beam of linearly polarized incident light is given by

$$T = k_1 \cos^2\theta + k_2 \sin^2\theta \qquad (3\text{-}10)$$

where θ is the angle between the plane of polarization of the incident beam (more accurately, the plane of the electric field vector of the incident beam) and the plane of preferred transmission of the polarizer. The orientation of the latter plane is usually clearly marked by engravings on the ring in which the polarizer is mounted. k_1 and k_2 are the principal transmittances of the polarizer, and

both are functions of the wavelength. Ideally, $k_1 = 1$ and $k_2 = 0$, but in reality k_1 is always somewhat less than unity, and k_2 always has some small but nonzero value.

In our design, we have an unpolarized beam and the angle θ is redefined to be the angle between the planes of preferred transmission (planes of polarization) of two sheet polarizers in near contact. It can easily be shown that the transmittance of the pair is given by

$$T_{pair} = k_1 k_2 \sin^2\theta + \frac{1}{2}\left(k_1^2 + k_2^2\right)\cos^2\theta \tag{3-11}$$

If we define

$$H_\perp = T_{pair}(\perp) = k_1 k_2 \qquad \text{and} \qquad H_{||} = T_{pair}(||) = \frac{1}{2}\left(k_1^2 + k_2^2\right) \tag{3-12}$$

Eq. (3-11) can be simplified to

$$T_{pair} = H_\perp \sin^2\theta + H_{||}\cos^2\theta = H_\perp + \left(H_{||} - H_\perp\right)\cos^2\theta \tag{3-13}$$

The quantity $H_\perp$ is called the closed transmittance or extinction ratio, while the quantity $H_{||}$ is called the open transmittance. Both quantities are wavelength-dependent, as is shown in the graphs. Because of the large ranges of open and closed transmission, it is convenient to plot the optical densities corresponding to these transmissions, rather than transmissions themselves. The open and closed optical densities are defined as follows:

$$D_{||} = \log\left(\frac{1}{H_{||}}\right) \quad \text{and} \quad D_\perp = \log\left(\frac{1}{H_\perp}\right) \tag{3-14}$$

Figure 3-13 shows the spectral dependence for the two orientations discussed above. The optical density of more than 4.0 can provide sufficient separation between these orientations with an error less than 0.1%.

$$\varepsilon < 10^{D_\perp(\lambda) - D_{||}(\lambda)} \approx 0.001$$

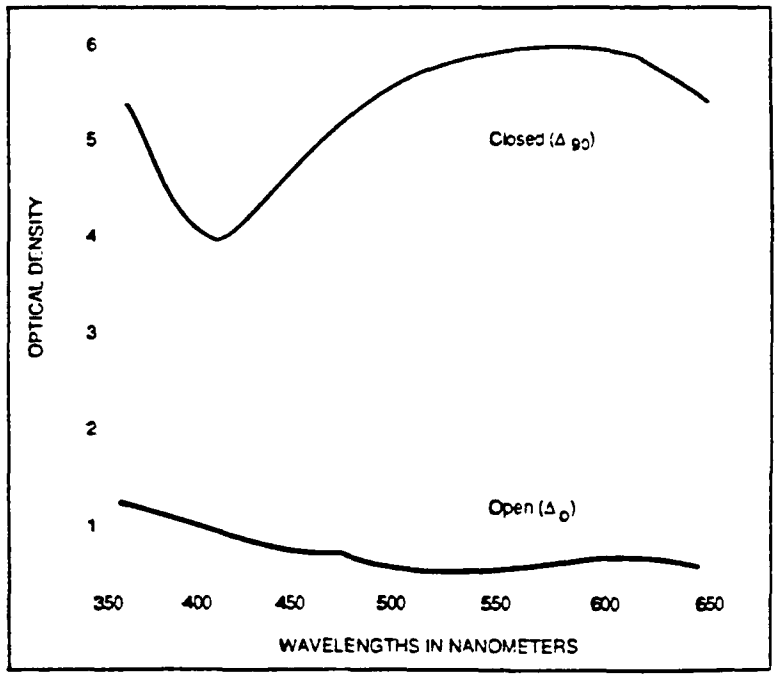


Figure 3-13 Performance of dichroic polarizers.

5. *Photomultiplier*

An Oriel photomultiplier was used with a sensitivity peak at $\lambda = 420$ nm. It was found that the dynamic range of the detected signal is less than one order and can be properly adjusted by using a neutral density filter.

3.3 Investigation of A Single-Block Highly-Compact Design of Polarization Fluorometer for AIDS Test

Based on POC's advanced technologies in interconnect and holography, we have studied several new designs. These designs will be a next generation fluorometer that will be mass producible, compact and cost effective. This design is schematically illustrated in Figure 3-14. Three of the most innovative features that highlight our design are:

1. The interference filter of POC's Phase I fluorometer design (Figure 3-8) can be replaced by two holographic concave gratings which will provide over twice the light intensity than is possible with interference filters. This allows us to use a smaller Xe-lamp.

2. To avoid the use of polarizers and the tuning operation, POC takes advantage of a substrate mode waveguide and decoupling surface hologram. This idea is based on POC's recent breakthrough in waveguide technology [29] and optical interconnects. Bouncing inside the substrate, as shown in Figure 3-14, the light pencil will gain preferable polarization in the direction parallel to the surface of the waveguide. By decoupling the guided wave with POC's unique hologram decoupling technique, further enhancement in degree of polarization is achieved. This enhancement in degree of polarization is clearly demonstrated in Figure 3-15. For example, if a hologram is made on a filter with proper thickness (say, around 12 μm), the diffraction efficiency (i.e., the intensity of light coming out of substrate and illuminating the sample) reaches maximum for one polarization while being zero for the perpendicular polarization.

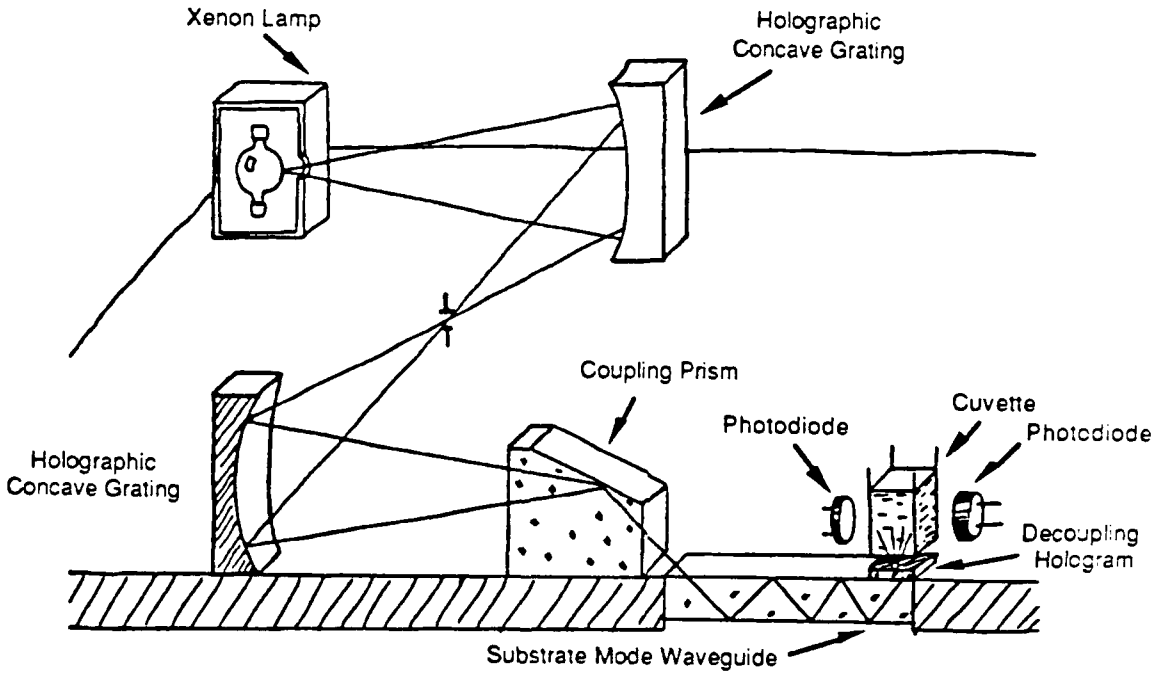


Figure 3-14 Schematic illustration of POC's next generation planar fluorometer for AIDS virus or antibody detection.

3. The detecting part of the polarization fluorometer can be simplified by using semiconductor photodetectors instead of photomultiplier tubes. Recent advances in highly sensitive photodiodes give us an opportunity to further reduce the weight and size of our proposed design (Figure 3-16). It may also be possible to have photodiodes with polarization selectivity. As a result, no output polarizers would be necessary for registration of fluorescence anisotropy. The device integration is very simple. Being equipped with a supporting microcontroller (not shown in Figure 3-14) the proposed model will be only 5" x 5" x 3" in size. Such a self-contained, easy to handle device is extremely desirable for in situ diagnostic of AIDS virus.

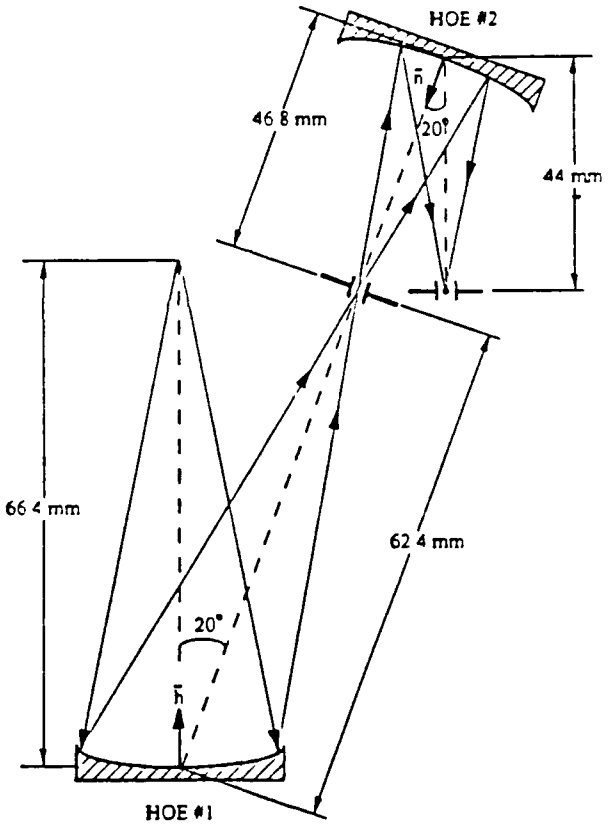


Figure 3-15 POC's concave holographic grating that combines spectral dispersion with light focusing capabilities that can be used in POC's new fluorometer design.

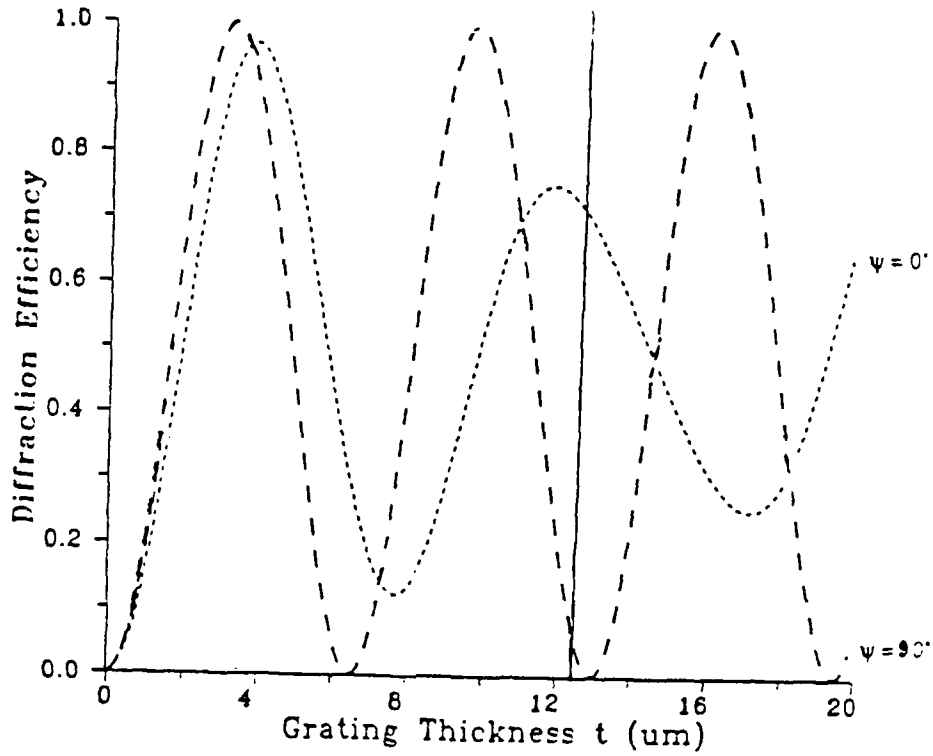


Figure 3-16 Plot of diffraction efficiency versus grating thickness at two perpendicular polarizations (Ref. [33]).

3.4 Review of Existing Technology

AIDS Detection Methods

Two routinely used methods of AIDS detection are the enzyme linked immuno assay (ELISA), and Western Blot. Both of these tests are antibody detection methods.

ELISA tests are available from several commercial suppliers (Abbott, Du Pont, Roche, Hoechst), each with their own levels of sensitivity and specificity.These tests use inactivated antigen reacted with T-lymphocytes which are coated on polystyrene beads or welled strips. Blood serum samples containing the HTLV antibody are coated onto the antigen preparations. Antibodies are detected by an enzyme linked antihuman Immunoglobulin (IgG).

Santos and Castro [30] have evaluated six commercially available ELISA tests. Although the tests were shown to be 98 - 100 % sensitive, the specificity for AIDS virus was relatively low. Because of this poor test specificity, false positive results occur (i.e., tests indicate HIV infection for healthy individuals). Currently ELISA tests are used as initial screening tests in routine clinical diagnosis. A positive test result requires two additional ELISA tests to be performed. If either of these further tests are positive also, the sample is designated as repeatedly reactive and requires further confirmation by Western Blotting technique. This process is time consuming, costly, and requires highly qualified and experienced operators. Further inadequacies of this test process arise due to the fact that ELISA tests detect HIV antibodies and not virus. It has been shown [31] that there is a 'window period' of three weeks or longer in which ELISA methods do not detect HIV antibodies in infected patients. This low sensitivity results in false negative results and hinders early disease detection.

The Western Blot procedure involves the incorporation of inactivated antigen into human T-cells. Proteins are separated by gel electrophoresis and incubated overnight with plasma samples. The antigen-antibody interaction is detected through the use of peroxidase labelled anti human globulin enzymes. Although Western Blot testing is reliable it has some major disadvantages; it is time consuming and requires considerable technical expertise, both in the performance of the test and in interpretation of the staining reactions. In addition, the sensitivity is dependent on both the quality of the antigen and the antibody detection reagents. Better standardization of the Western Blot test procedure concerning the interpretation of banding patterns is anticipated.

Other diagnostic tests have been developed, such as the radioimmunoprecipitation test (RIP) and hemagglutination, but these have found less use in clinical diagnosis procedures.

Fluorescence Detection Methods

The indirect immunofluorescence (IIF) technique has been reported to provide sensitive and specific detection of HIV antibody.

Indirect immunofluorescence (IIF) has been investigated for use in AIDS detection recently. Indirect detection is accomplished by incorporating a fluorescent compound, such as fluorescing

isothiocyanate, with immunoglobulins (IgG). This solution is used to stain sample cell suspensions spotted on microscope slides which are then UV illuminated. The antibody end point is taken as the highest dilution which shows specific nuclear fluorescence. IIF has been reported to provide sensitive and specific detection of HIV antibodies.

IIF techniques, like ELISA tests, are not able to directly detect virus antigens. The utility of the IIF tests has been questioned, since the discovery that immunoglobulin G receptor sites, induced in cytoplasmic cells may produce false-positive results due to nonspecific staining [32].

Anticomplementary immunofluorescence (AIF) has been reported to detect both circulating antibodies and cell-associated viral antigens in the detection of human T-cell lymphotrophic virus (HTLV-III) [33] and cytomegalovirus (CMV) [34]. AIF involves the use of a fluorescent dye conjugated with the IgG fraction of antiserum to complement cells. To date, AIF techniques have not been reported in use for the detection of the HIV virus.

Experimental Analysis of Chemical Probes

Because no single probe compound can adequately respond to the changes in a variety of chemical environments, the design and synthesis of probe compounds is very important in biosensor applications. Candidate compounds need to be designed and evaluated in model environments to assess their utility in biological systems. Chemical analysis of synthesized compounds must be performed to determine the exact structure of the products. Typical chemical analysis techniques can be employed to some extent in verifying the structure of probe compounds. Characterization by ultraviolet absorption spectroscopy (UV) indicates the type of light absorbing units (chromophores) present in a molecule. UV spectroscopy allows one to determine the optimum wavelength for excitation of the probe to induce fluorescent behavior. Lower energy excitations of chemical compounds induces symmetrical and asymmetric bending and vibrational motions. Thus infrared (IR) light can be used to excite the molecules and subsequently identify specific functional groups present in the molecule. IR spectra can be performed on very small amounts of liquid samples or solids using POC's newly acquired Perkin-Elmer 1620 FT-IR. Solid opaque films can be analyzed using POC's Attenuated Total Internal Reflection (ATIR) apparatus. Nuclear magnetic resonance (NMR) can be employed to indicate the sequence of functional groups and their relationship with neighboring groups. Elemental analysis of the probes will provide the exact amount of each chemical element present, this in conjunction with the above information is very helpful in verifying the structure of the synthesized probe compound.

4.0 CONCLUSIONS AND RECOMMENDATIONS

During Phase I of this contract, POC has extensively studied the application of polarization fluorometers for fluorescence anisotropy of lipid probes for the detection of biological agents. Specifically, this technique was applied to recognize the presence of PBMC and the antibody to T-cell. Also investigated were the chemical probes, new and improved fluorometer designs, and fabrication of a unique device that will be ultimately used in the hospitals and clinics for the diagnosis of AIDS patients. Based on our six month investigation, we have drawn certain conclusions and have made recommendations for further directions that we consider are crucial for the early detection of this deadly disease. These conclusions and recommendations are:

1. The fluorescent probes used by POC are unique and the diagnostic test based on these chemical probes can be an alternative to ELISA or Western Blot tests that are currently used.

2. Our results of the Phase I investigation support the fact that polarization fluorometer for fluorescence of anisotropy of lipid probe is a unique way of monitoring ligand-receptor interactions in various biological systems. Such ligand-receptor interactions have previously been used to monitor toxins [6], drugs [4], and antibodies [7].

3. POC's PFFALP method has several such advantages such as (i) high sensitivity, (ii) specific response, (iiv) speed, (iv) size, and (v) cost effectiveness.

4. Both (ASM) and (CAPC) can be obtained in pure chemical compositions, which, however, need to be characterized in order to understand the morphological factor that may be responsible for the fluorescence polarization changes in the presence and absence of the biological agent, antigen, or antibody.

5. We have investigated several designs and have concluded that the compact fluorometer design based on POC's interconnect and advanced holographic technology may be a much more suitable design for a future compact fluorometer device for AIDS diagnosis.

6. We need to develop a systematic protocol, in collaboration with Harbor-UCLA Medical Centers' Dr. Gildon Beal, with patients so that routine tests can be conducted twice in a week in the next phase of this contract.

7. The stability of fluorometer and its reliability need to be tested by continuing a series of tests that have been initiated during Phase I. In addition tests should be reported by using different blood groups as well as low to high levels of infected sera in order to confirm results.

8. We conclude that PFFALP has the merit to be considered as a new AIDS diagnostic test device, however, additional team efforts involving medical doctors, immunologists, chemists, and spectoscopists are required.

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